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ACTIVATION OF INNATE IMMUNITY BY 14-3-3 ε, A NEW POTENTIAL ALARMIN IN OSTEOARTHRITIS

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Abstract

Objective: The innate immune system plays a central role in osteoarthritis (OA). We identified 14-3-3ε as a novel mediator that guides chondrocytes toward an inflammatory phenotype. 14-3-3ε shares common characteristics with alarmins. These endogenous molecules, released into extracellular media, are increasingly incriminated in sustaining OA inflammation. Alarmins bind mainly to TLR2 and TLR4 receptors and polarize macrophages in the synovium. We investigated the effects of 14-3-3ε in joint cells and tissues and its interactions with TLRs to define it as a new alarmin involved in OA.

Design: Chondrocyte, synoviocyte and macrophage cultures from murine or OA human samples were treated with 14-3-3ε. To inhibit TLR2/4 in chondrocytes, blocking antibodies were used. Moreover, chondrocytes and bone marrow macrophage (BMM) cultures from KO TLRs mice were stimulated with 14-3-3ε. Gene expression and release of inflammatory mediators (IL-6, MCP-1, TNFα) were evaluated via RT-qPCR and ELISA.

Results: *In vitro*, 14-3-3ε induced gene expression and release of IL6 and MCP1 in the treated cells. The inflammatory effects of 14-3-3ε were significantly reduced following TLRs inhibition or in TLRs KO chondrocytes and BMM.

Conclusions: 14-3-3ε is able to induce an inflammatory phenotype in synoviocytes, macrophages and chondrocytes in addition to polarizing macrophages. These effects seem to involve TLR2 or TLR4 to trigger innate immunity. Our results designate 14-3-3ε as a novel alarmin in OA and as a new target either for therapeutic and/or prognostic purposes.

1 **Keywords :** 14-3-3 ε, alarmin, osteoarthritis, Innate immunity, TLR, synovitis

2 **Running headline :** 14-3-3 ε : a new alarmin in osteoarthritis

3

4 **Abbreviations**

5 BMM: bone marrow derived macrophages

6 BSA: bovine serum albumin

7 cDNA: complementary deoxyribonucleic acid

8 DAMP: damage-associated molecular pattern

9 DMEM: Dulbecco's modified Eagle's medium

10 EDTA: Ethylenediaminetetraacetic acid

11 ELISA: enzyme linked immunosorbent assay

12 FBS: fetal bovine serum

13 FLS: Fibroblast-like synoviocyte

14 Glu: glutamine

15 H&E: Hematoxylin and eosin

16 HPRT: Hypoxanthine-guanine phosphoribosyltransferase

17 IL : interleukin

18 KO: Knockout

19 LPS: lipopolysaccharides

20 mAB: monoclonal antibody

1   MCP1 : monocyte chemoattractant protein-1

2   MMP: matrix metalloproteinases

3   mRNA: messenger ribonucleic acid

4   OA: osteoarthritis

5   OxPAPC: Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine

6   PBS: phosphate buffered saline

7   PRRs: pattern-recognition receptors

8   PS: penicillin/streptomycin

9   RA: rheumatoid arthritis

10   RNA: ribonucleic acid

11   RT-PCR: real-time polymerase chain reaction

12   TLR: toll like receptor

13   TNFα: Tumor necrosis factor alpha

14   WT: wild-type

15

Introduction

Osteoarthritis (OA) is a highly complex and the most prevalent joint disorder with a total of 242 million people affected worldwide[1]. Clinical symptoms include severe pain, joint stiffness and reduced function, which seriously decrease quality of life[1]. Unfortunately, only a few drugs are weakly effective for treating symptoms, and no disease-modifying osteoarthritis drugs (DMOADs) are available to date. Initially considered cartilage driven, OA is a much more complex disease with inflammatory mediators released by cartilage, bone and synovium[2]. Increasing evidence suggests that inflammation is present in OA and has raised the possibility that inflammation and the innate immune system could be active players in the development and progression of OA [3,4]. Innate immune responses involve the activation of resident leukocytes such as macrophages, production of inflammatory mediators (cytokines, chemokines, and lipid metabolites), recruitment of neutrophils and monocytes/macrophages, and aims to eliminate invading microorganisms and injured tissues[5]. Histopathological studies have confirmed that immune cell infiltration is extremely common in OA histological specimens[6]. The innate immune system participates in inflammation triggered by host molecules or fragments collectively called DAMPS (danger-associated molecular patterns) or alarmins [5,7,8].

The term "alarmin" was proposed by Oppenheim and co-workers in 2005 to classify proteins that are rapidly released during infection or tissue damage, activating immune cells after interaction with their specific receptors[7]. Alarmins are now considered to be markers of destructive processes in joints[9]. Because of their fast release as a result of cell stressor nonprogrammed cell death, alarmins are among the first factors to be secreted and, as such, act as first responders to stimuli.

In addition to their role in disease initiation, alarmins also amplify and sustain inflammatory processes and, thus, play a notable role in the pathogenesis of inflammatory conditions[10,11]. A number of alarmins have been detected at high levels in OA tissues and synovial fluid, including HMGB1, UA, ATP, thymosin ß4 and various S100 proteins, enhancing catabolic processes and inflammatory responses that contribute to disease progression[8,12–14].

Synovitis, the inflammation of the synovium, can occur in early stages of OA[15]. OA synovitis directly contributes to several clinical signs and symptoms including joint swelling and effusion, and reflects the structural progression of the disease[15,16]. Moreover, synovitis is significantly associated with OA severity[17]. Macrophages are the main immune cell type in the healthy synovium and are likely the front-line cells that sense joint damage. These cells also contribute to OA progression in response to alarmins by producing MMPs and cytokines[18]. The main morphological characteristic of synovitis is macrophage accumulation in the intimal lining[19]. Macrophages are characterized by heterogeneity and plasticity in response to stimuli from their microenvironment, leading to a continuum of phenotypes where M1/M2 are the 2 extremes [20]. M1 macrophages are activated by interferon-γ and lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNF-α), resulting in the secretion of large amounts of proinflammatory cytokines and mediators such as TNF-α, interleukin (IL)-1 and IL-6[21]. M2 macrophages have been further divided into specific subtypes and possess anti-inflammatory activity[22]. Studies have shown that macrophages accumulate and become polarized (M1 or M2) in the synovium during OA development [23]. The classification of macrophages into M1/M2 subtype is reductive. Recently, the heterogeneity of macrophage phenotypes in OA patients has

been studied and revealed a more complex classification [24]. However, the role of macrophages, their polarization in OA development and the underlying mechanisms are still unknown.

Activated synovial cells (synoviocytes or macrophages) secrete several degenerative enzymes and inflammatory mediators, as well as alarmins [14,25]. These alarmins activate pattern-recognition receptors (PRRs) including TLRs (toll-like receptor) in the OA-affected cartilage and synovium [26,27], which in turn amplifies inflammation and degeneration of cartilage. TLRs are transmembrane receptors that display binding affinity for a variety of DAMPs. According to their ligands, location and signaling pathways, ten different genes can be distinguished in humans [28]. TLRs activate signaling pathways that result in the production of cytokines, chemokines, and various inducible molecules associated with the immune response. TLR2 and TLR4 are overexpressed in OA cartilage, and their presence correlates with histopathological damage [29,30].

Our team identified 14-3-3ε as a novel soluble mediator that is critical in the communication between subchondral bone and cartilage in OA [31]. The 14-3-3 proteins, a family of seven isoforms (β, ε, γ, η, θ, σ, ξ), are involved in a wide range of vital regulatory processes by binding to more than 200 intracellular proteins [32]. Under normal conditions, these proteins reside intracellularly; however, some reports indicate its presence extracellularly. Externalization of 14-3-3 appears to be mediated by a non-classical pathway, similar to IL1-β, due to the absence of a signal peptide sequence [33]. 14-3-3 proteins are released into the extracellular space through an exosomal pathway [34]. Extracellular 14-3-3s are now thought to play an important role in the pathogenesis of certain inflammatory conditions. In OA, our team showed that 14-3-3ε, released by osteoblasts in response to mechanical stress, skews

7

chondrocytes toward a pro-catabolic phenotype by strongly inducing the expression of MMPs in an CD13/APN-dependent manner [31,35]. 14-3-3ε seems to share common characteristics with other alarmins (such as ATP and thymosin β4) in inducing degradation of the cartilage matrix[8]. Extracellular 14-3-3ε proteins could, therefore, be classified as alarmins that are derived from activated or damaged osteoblasts.

Our aim is to investigate the effects of 14-3-3ε on the different cell types of the joint and its role in the activation of innate immunity by studying interactions with its potential receptors TLR2 and TLR4.

Methods

Materials

All reagents were purchased from Sigma-Aldrich (Lyon, France), unless stated otherwise. Fetal bovine serum (FBS) was obtained from Invitrogen (Cergy-Pontoise, France). Liberase TM and complete protease inhibitor mixture were from Roche Diagnostics (Meylan, France). Recombinant human 14-3-3ε was from Enzo Life Sciences. Anti-TLR2 antibody and OXPAPc (oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine) were from InvivoGen (Toulouse, France). Anti-TLR4 antibody was from Santa Cruz Technology (Heidelberg, Germany.

Collection of human OA synovium

Human OA knee explants were obtained from patients undergoing total knee arthroplasty due to OA at Saint-Antoine Hospital (Paris) or at the Maussins clinic (Paris) (BioJOINT, a biobank of OA human knee , legal authorization: CPP Paris Ile de France V, CNIL reference: MMS/ HGT/AR177404). Informed consent for the use of tissue and clinical data was obtained from each patient before surgery. Experiments with human samples were approved by a French Institutional Review Board (Comité de Protection des Personnes, Paris Ile de France V and Commission Nationale de Informatique et des Libertés).

1

Mice

3   Mice on a C57BL/6 J background, 8–12 weeks old, were used in all experiments.

4   Wild-type (WT) mice were purchased from Janvier Laboratories. The animal housing

5   facility was granted approval (C 75-12-01) by the French Administration. All

6   experiments were conducted according to the European Communities Council

7   Directive (2010/63/UE) and approved by the Regional Animal Care and Use

8   Committee (Ile-de-France, Paris, n°5; agreement number 00917.02 and 4625).

9   *Tlr2−/−*, and *Tlr4−/−* mice were kind gifts from Professor Shizuo Akira (Osaka

10 University, Japan) and with the collaboration of Professor F. Pene (Institut Cochin,

11 France). All knockout (KO) were maintained in the specific pathogen-free (SPF)

12 animal facility of the Cochin Institute.

13

Cell cultures

15 Detailed protocols are described in supplemental data and (36).

16 *Synovial explants :* For explants, synovium from OA patients was cut into small

17 pieces aseptically and incubate in 24-well plate before treatment.

18 *Primary culture of synovial fibroblasts:* Primary cultures of fibroblast like synoviocytes

19 are obtained after enzymatic digestion (collagenase and DNase) of human synovial

20 membrane samples (Biojoint biobank). The digestion solution was then placed in

21 culture flasks during an overnight incubation. The digestion solution was removed,

22 and adherent cells were washed 3 times with phosphate buffered saline (PBS) and

23 cultured in FLS growth medium at 37°C in a humidified atmosphere (5% CO_2). After

1 reaching their confluence, FLS were counted and cultured in 12-well plates for

2 stimulations [37].

3 *Primary culture of murine articular chondrocytes:* Mouse primary chondrocytes were

4 isolated from the articular cartilage of 5 to 6-day-old C57Bl6 mice from Janvier (St.

5 Berthevin, France). All experiments were performed according to protocols approved

6 by the French and European ethics committees (Comité Régional d'Ethique en

7 Expérimentation Animale N°3 de la région Ile de France). Each littermate among the

8 mice was used for one experiment. After 1 week, the cells were incubated in fasting

9 medium for 24h before treatment.

10 In addition, experiments were also performed using *TLR2−/−* and *TLR4−/−* mice.

11 *Primary culture of murine bone marrow mononuclear (BMM) cells:* Bone marrow

12 mononuclear (BMM) phagocytic precursor cells were isolated from femurs and tibiae

13 of *WT* and *TLR2−/−* and *TLR4−/−* mice. These precursors were differentiated into

14 adherent mature macrophages (BMM) for 7 days in complete medium containing 10

15 ng/mL of macrophage colony stimulating factor (PeproTech, Neuilly-sur-Seine,

16 France).

17 *Culture of the THP-1 cell line:* Human monocytic THP1 cells (American Type Culture

18 Collection, Rockville, MD, USA) were kind gifts from Professor Rouis (Sorbonne

19 University, France). Cells were routinely grown to a primary macrophage culture

20 following differentiation from monocytes with a 24h treatment with Phorbol 12-

21 Myristate 13-Acetate (50 nM) PMA, Sigma, Saint-Louis, USA). After 72h, the

22 macrophages were starved before treatment.

1   *Treatment by recombinant 14-3-3ε:*   All cell cultures were stimulated with

2   recombinant 14-3-3ε at 1 µg/ml for 24h. Supernatants and total mRNA collected after

3   cell lysis were harvested and stored at -80°C.

4   For blocking antibody and pharmacological experiments, murine articular

5   chondrocytes were pretreated for 20 minutes with increasing concentrations (1 and 5

6   µg/ml) of a mouse TLR2 or TLR4 antibody or oxidized 1-palmitoyl-2-arachidonoyl-sn-

7   glycero-3-phosphocholine (OxPAPC) at 0.3, 3 and 30 µg/ml before the treatment by

8   recombinant 14-3-3ε.

9

RNA extraction and quantitative RT-PCR

11   Total RNA was extracted from murine chondrocytes using the ReliaPrep RNA Cell

12   Miniprep System kit (Promega, Madison, WI, USA) from human synovial fibroblasts,

13   BMM and THP1 by Trizol chloroform. Concentrations were determined by

14   spectrophotometry (Eppendorf, Le Pecq, France). Reverse transcription was

15   performed with 500 ng of total RNA with the Omniscript RT kit (Qiagen). mRNA levels

16   were quantified with the Light Cycler LC480 (Roche Diagnostics, Indianapolis, IN,

17   USA). PCR amplification conditions are described in supplemental data. Product

18   formation was detected at 72°C in the fluorescein isothiocyanate channel. The mRNA

19   levels were normalized to those of murine HPRT or Human 18S. Specific primer

20   sequences are presented in Table S1.

21

Protein secretion quantification by ELISA

23   Total mouse and human IL6, MCP1, TNFα, MMP-3 and 14-3-3ε secretion were

24   assayed in cell-free supernatants using an enzyme-linked immunosorbent assay

(ELISA) kit (R&D Systems and Abbexa) according to the manufacturer's instructions. Concentrations were analyzed in duplicate at serial dilutions and determined by comparison against a standard curve.

Endotoxin tests:

Protocol of the experiments is described in supplemental data

Statistical Analysis

The choice of the number of experiments was established by power analysis tests and previous and published work from our laboratory. The small number of experiments used for each part of the work can be considered as a limitation. All data were showed as mean values +/- s.e.m. In Fig. 5a and b, the stimulated condition (14-3-3ε) was normalized to 1 in order to study the inhibition rates and data were showed as mean values with 95% confidence intervals (CI). Statistical analyses were performed with the Mann Whitney test to compare mean values between 2 groups or by the Wilcoxon test when analyses were based on patient paired-matched samples (Fig.1 and 2). One-way analysis of variance (ANOVA) and two-way ANOVA with the Bonferroni multiple comparisons post-test were used to compare mean values between more than 2 groups using GraphPad Prism software (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ns: not significant.

Results

Stimulation of synovium explants from OA patients by 14-3-3ε elicits the release of pro-inflammatory factors.

To verify whether 14-3-3ε is able to induce synovium inflammation in humans, we used synovium explants from OA patients to mimic the pathophysiological environment as closely as possible. Stimulation of these human synovium explants with recombinant 14-3-3ε (1 µg/ml) induced the secretion of MCP1 and IL6 protein (Fig. 1A, B). Indeed, mean difference of IL6 protein release showed a 5.7-fold increase between control and 14-3-3ε stimulation (0.6, 95%CI [0.3;0.9] vs 3.4, 95%CI [2.7;4.1] respectively) (Fig. 1A) and mean difference of MCP1 protein release showed a 9.5-fold increase between control and 14-3-3ε stimulation (4.8, 95%CI [0.9;8.6] vs 45.6, 95%CI [9.9;81.3] respectively) (Fig. 1B).

14-3-3ε elicits a pro-inflammatory phenotype in fibroblast-like synoviocytes (FLS).

To more precisely study the impact of 14-3-3ε on the two main cell types residing in the synovium, FLS and macrophages, we stimulated primary cultures of FLS from OA patients with 14-3-3ε recombinant protein (1 µg/ml) for 24h. The levels of IL-6 and MCP1 in controls samples are below the detection threshold. We found that stimulated synoviocytes had increased mRNA expression and secretion of both IL6 and MCP1 (Fig. 2 A-D). Mean difference of IL6 mRNA expression showed an increase of 7.5 fold between control and 14-3-3ε stimulation (0.5, 95%CI [0;1.0] vs 3.7, 95%CI [2.1;5.3] respectively) and MCP1 mRNA expression showed an increase of 4.4 fold mean difference between control and 14-3-3ε (0.6, 95%CI [0.1;1.1] vs 2.6, 95%CI [0.9;4.3] respectively) (Fig. 2A, B). Mean difference of protein secretion of IL6

and MCP1 showed a fold increase of 3.7 (5.5, 95%CI [-0.6;11.6] vs 20.54 95%CI [-0.7;41.7]) and 31.8 (1.7, 95%CI [1.0;2.4] vs 54.2, 95%CI [32.7;75.7]) between control and 14-3-3ε stimulation respectively (Fig. 2C, D).

14-3-3ε skews macrophages toward a pro-inflammatory phenotype involving Toll-like receptors signaling.

Stimulation of human macrophages derived from the THP1 cell line with 14-3-3ε induced mRNA expression and secretion of both IL6 and MCP1 (Fig. 3A, B, D, E). Mean difference of mRNA expression levels of IL6 and MCP1 showed a fold increase of 7.2 (0.1, 95%CI [0;0.2] vs 0.7, 95%CI [0.5;0.8]) and 3.1 (0.2, 95% CI [0;0.4] vs 0.7, 95%CI [0.5;0.8]) between control and 14-3-3ε stimulation respectively. Similarly, the release of IL6, MCP1 and TNFα protein was increased in the supernatants of macrophages stimulated with 14-3-3ε, showing a fold mean difference of 17.8 (0.03, 95%CI [0;0.05] vs 6.2, 95%CI [1.9;10.4]), 18.9 (23.9, 95%CI [23;24.8] vs 453, 95%CI [379.8;525.6]) and 15.1 (0.04, 95%CI [0;0.07] vs 0.6, 95%CI [0.2;1.1]) between control and 14-3-3ε stimulation respectively (Fig. 3D, E, F). Moreover, macrophages subjected to 14-3-3ε stimulation displayed increased mRNA expression of CD38, another marker associated with the M1 pro-inflammatory phenotype, with a 6.6-fold mean difference between control and 14-3-3ε stimulation (0.1, 95%CI [0;0.2] vs 0.9, 95%CI [0.7;1.1]) (Fig. 3C),

To assess the implications of TLR2 and TLR4 in the cellular response to 14-3-3ε, we used primary cultures of bone marrow macrophages (BMM) from TLR2 or TLR4 KO mice and stimulated them with recombinant 14-3-3ε. Untreated WT, TLR2 KO and TLR4 KO BMM showed no protein release of IL6 and MCP1, whereas IL-6 ~~level~~ and

MCP1 release were increased by WT BMM cells stimulated with 14-3-3ε. IL-6 protein release was significantly decreased in the supernatants of TLR2 and TLR4 KO BMM stimulated with 14-3-3ε compared to the WT BMM stimulated with 14-3-3ε (64% inhibition for TLR2, WT 2.5, 95%CI [0.9;4.1] vs TLR2 KO 0.9, 95%CI [0.4;1.3] and 84% inhibition for TLR4; TLR4 KO 0.4, 95%CI [0.2;0.6]) (Fig. 4A). MCP1 showed the same tendency (22% inhibition for TLR2 WT 2.8, 95%CI [-2.0;7.7] vs TLR2 KO 2.2, 95%CI [-1.5;6.0] and 68% inhibition for TLR4; TLR4 KO 0.9, 95%CI [-2.1;3.8]) (Fig. 4B).

14-3-3ε elicits a catabolic and inflammatory phenotype in murine articular chondrocytes involving TLR2 and TLR4.

Murine chondrocytes were sensitive to stimulation with 14-3-3ε recombinant protein and showed increased mRNA expression and secretion of pro-catabolic (MMP3) and pro-inflammatory (IL6) factors.

To confirm the involvement of TLR2 and TLR4 in 14-3-3ε signaling, murine articular chondrocytes were pre-treated with specific TLR2, TLR4 blocking antibody or the pharmacologic inhibitor (OxPAPC) inhibiting both receptors followed by 14-3-3ε stimulation. MMP3 mRNA expression and protein secretion induced by 14-3-3ε were significantly and dose-dependently reduced by TLR4 blocking antibody treatment (MMP3 mRNA expression inhibition fold: 78%, (95%CI [-0.1;0.6]) (1 µg/ml) and 77% (95%CI [-0.02;0.5]) (5 µg/ml) (Fig. 5A); secretion: 69%, (95%CI [-0.18;0.81]) (1 µg/ml) and 84%, (95%CI [-0.09;0.42]) (5 µg/ml) (Fig. 5D)). Anti-TLR2 blocking antibody reproduced the same pattern (mRNA expression: 79%, (95%CI [-0.01;0.43]) (1

µg/ml) and 83%, (95%CI [0.04;0.30]) (5 µg/ml) (Fig. 5B); secretion 73%, (95%CI [-0.07;0.61]) (1 µg/ml) and 74%, (95%CI [0.04;0.48]) (5 µg/ml) (Fig. 5E)). Furthermore, the pharmacologic inhibitor OxPAPC, inhibiting both receptors, markedly decreased MMP3 mRNA expression and protein release in a dose-dependent manner (mRNA expression: from 71% (95%CI [0.04;0.54]) (0.3 µg/ml) to 98% (95%CI [-0.01;0.04]) (30 µg/ml) (Fig. 5C); secretion: from 64% (95%CI [0.14;0.58]) (0.3 µg/ml) to 96% (95%CI [-0.01;0.08]) (30 µg/ml) (Fig. 5F)).

Inhibition of TLR2 and/or TLR4 also impacted the mRNA and protein expression of the pro-inflammatory cytokine IL6 by murine chondrocytes. (IL6 mRNA expression: anti-TLR4 treatment: 73% (95%CI [-0.15;0.68]) (1 µg/ml) and 71% (95%CI [-0.10;0.69]) (5 µg/ml) (Fig. 5G); secretion: 79% (95%CI [-0.12;0.55]) (1 µg/ml) and 81% (95%CI [-0.10;0.48]) (5 µg/ml) (Fig. 5J); anti-TLR2 treatment: IL6 mRNA expression 53% (95%CI [-0.02;0.97]) (1 µg/ml) and 51% (95%CI [0.04;0.94]) (5 µg/ml) (Fig. 5H); secretion 69% (95%CI [-0.03;0.64]) (1 µg/ml) and 78% (95%CI [0.01;0.43]) (5 µg/ml) (Fig. 5K)). Inhibiting both receptors simultaneously with OxPAPC resulted in decreases IL6 expression and secretion (IL6 mRNA expression: from 69% (95%CI [0.01;0.60]) (0.3 µg/ml) to 92% (95%CI [0;0.15]) (30 µg/ml) (Fig. 5I); secretion: from 69% (95%CI [0.05;0.58]) (0.3 µg/ml) to 96% (95%CI [-0.02;0.09]) (30 µg/ml) (Fig. 5L). Stimulations of chondrocytes with the different treatments showed high variability between cultures.

To confirm the results obtained in KO BMM, we also stimulated TLR2 or TLR4 KO murine articular chondrocytes with 14-3-3ε. TLR2 and TLR4 KO murine chondrocytes exhibited decreased mRNA expression and protein release of IL6 but also mRNA expression of MMP3 and MMP13 compared to WT chondrocytes (Fig. 6). IL6 mRNA expression was significantly decreased in TLR2 and TLR4 KO chondrocytes treated

with 14-3-3ε compared to the WT chondrocytes (75% for TLR2; WT 65, 95%CI [-53.7;183.8] vs TLR2 KO 16.5, 95%CI [3.6;29.4] and 97% for TLR4; TLR4 KO 1.8, 95%CI [1.0;2.5]) (Fig. 6B), and its release was greatly attenuated in TLR2 (77% for TLR2 (WT 21.8, 95%CI [-1.5;45.1] vs TLR2 KO 5.0, 95%CI [2.0;8.0]) and TLR4 (by 97% for TLR4, TLR4 KO 0.7, 95%CI [0.5;0.9]) KO chondrocytes in response to 14-3-3ε stimulation respectively (Fig. 6A). Similarly, MMP3 and MMP13 mRNA expression levels were also decreased in TLR2 and TLR4 KO chondrocytes (For MMP3: 60% for TLR2, WT 988, 95% CI [-588;2566] vs TLR2 KO 394.1, 95%CI [242.3;545.8] and 94% for TLR4; TLR4 KO 61.4, 95%CI [46.9;75.9]; For MMP13: 22% for TLR2, WT 0.5, 95% CI [0;1] vs TLR2 KO 66.4, 95%CI [48.6;84.2] and 77% for TLR4; TLR4 KO 20.2, 95% CI [10.4;30.1]) (Fig. 6C and 6D). Taken together, these results demonstrate the involvement of both TLR2 and TLR4 receptors in 14-3-3ε signaling.

Discussion

We have recently identified 14-3-3ε as a new soluble mediator involved in deleterious biochemical interactions between bone and cartilage [30]. In the present study, we showed that 14-3-3ε is a new alarmin and can act particularly in the synovium during OA pathogenesis. Its role in the activation of innate immunity leading to synovitis could be due to interactions with its potential receptors, TLR2 and TLR4.

It is now established that up to 50% of OA patients have synovitis, as demonstrated by magnetic resonance imaging, ultrasonography and arthroscopy [38]. Based on these results and to study the involvement of 14-3-3ε in the synovium of

the OA joint, we analyzed the effects of 14-3-3ε on whole synovial tissue explants

from OA patients by measuring the release of pro-inflammatory factors. We found

that IL6 and MCP1 release in culture media was significantly increased after 14-3-3ε

stimulation compared with the control explants. In these complete synovial explants

from OA patients, the pathophysiological environment was conserved. There was

obvious heterogeneity in the degree of inflammation of the synovium between patient

samples, which could be due to the different pathological mechanisms leading to

OA among patients[39,40]. Although fewer synovial macrophages are present in OA

compared with RA, they are crucial for the production of proinflammatory cytokines

such as IL6[18]. Previous studies have shown that selective depletion of synovial

macrophages during experimental OA largely reduces cartilage damage and

osteophyte formation, which are 2 major hallmarks of OA[41]. Thus, we would like to

separately analyze the role in synovitis of 2 main cell types residing in the synovium:

fibroblast-like synoviocytes (FLS) and macrophages. We found that 14-3-3ε was able

to increase the mRNA expression and protein secretion of IL6 (7.5 and 4.3-fold

increase respectively) and MCP1 (4.4 and 32-fold increase) in FLS. These cells were

able to respond to 14-3-3ε resulting in an inflammatory phenotype. Moreover, Thp1

cells, a human monocyte cell line, were used to address whether 14-3-3ε could

polarize these cells toward a pro-inflammatory macrophage phenotype. In our current

study, we showed that 14-3-3ε increased the mRNA expression and protein secretion

of IL6 (7.2 and 17.8-fold increase respectively) and MCP1 (3.1 and 19-fold increase).

In addition to the experiments on Thp1, we also performed stimulations of primary

cultures of murine macrophages (Bone marrow macrophages: BMM) by 14-3-3ε and

studied the expression of different markers. mRNA expressions of pro-inflammatory

mediators such as iNOS, IL-1β and TNFα were increased whereas mRNA

expression of anti-inflammatory marker (EGR2) was decreased in BMM (Fig. S1 Supplemental data). This protein subsequently appears to polarize macrophage toward the M1 phenotype. In the case of knee OA, a study analyzing M1 macrophages and M2 macrophages in synovial fluid in normal versus OA knees found a higher ratio of M1/M2 in OA versus normal knees, and the ratio was significantly correlated to the Kellgren-Lawrence grade[42]. These results suggest that macrophage polarization may indeed play a role in the control and even progression of OA disease. However, it should be noted that the classification of macrophages into M1/M2 subtype is reductive. This ability of macrophages to modify their phenotype in response to external signals gives rise to a broad spectrum of possibilities depending on their interactions[24]. Our study indicates that 14-3-3ε can induce a proinflammatory environment in the OA synovium through stimulation of macrophages which possibly contributes to the joint destruction that occurs during OA. Similar results have been obtained previously for the alarmin S100A9 in the synovium[43].

Interestingly, high levels of many alarmins have been described in the synovial fluid of OA patients[12]. Numerous studies have shown that these DAMPs stimulate synovial cell proliferation, influence hypertrophic chondrocyte differentiation and induce inflammatory and pro-catabolic events in vitro, and they promote synovitis and cartilage degradation *in vivo* in murine OA models[44]. We hypothesized that 14-3-3ε is a new alarmin involved in OA. Our results showed that this protein was able to lead to an inflammatory and catabolic response in joint similarly to other alarmins. Moreover, OA is mainly linked to activation of innate immunity by the binding of damage-associated molecular patterns (DAMPs) to so-called pattern recognition receptors (PRRs)[45]. Of central importance in the PRR family are the Toll-like

receptors (TLRs)[45]. TLR2 and TLR4 are overexpressed in OA cartilage, and their presence correlates with histopathological damage[29]. Blockade of TLR signaling -as shown in TLR2/TLR4 and in MyD88 knockout mice- downregulates cartilage catabolic response *in vitro*, and it can protect animals from experimental OA[46]. Moreover, synovial fluid proteins from patients with OA activate macrophages via TLR2/TLR4 receptors, in turn translocating NF-κB to the nucleus[9]. Synovial fibroblasts are sensitive to both mechanical alterations and DAMPs due to the expression of different TLRs on the cell membrane, resulting in increased synthesis of pro-inflammatory mediators[27]. In particular, TLR-2 and TLR-4 are used by many alarmins[47].

To examine whether 14-3-3ε response was driven by TLR, we used TLR2 and TLR4 KO mice to study their potential involvement in macrophage and chondrocyte responses. Our results showed that these two receptors were involved in the inflammatory and catabolic phenotype after stimulation with 14-3-3ε, with TLR4 showing predominant involvement. To further investigate the involvement of TLR2 and TLR4, we used blocking antibodies against them and a pharmacological inhibitor (OXPAPC) that is able to inhibit both TLR2 and TLR4. Our results validated the results obtained in the KO mice and confirmed that 14-3-3ε could elicit a catabolic and inflammatory phenotype in murine articular chondrocytes and a pro-inflammatory phenotype in BMM macrophages. Interestingly, a TLR4 monoclonal antibody has recently been demonstrated to have an adequate safety profile, and a phase II clinical trial in patients with RA has been launched[48].

In the present study, we used recombinant 14-3-3ε protein produced in E*scherichia coli*, similarly to many commercially available recombinant proteins. Although this expression system has many advantages, including rapid expression,

high yields, ease of culture and low cost[49], the proteins recovered may be contaminated with endotoxin, a highly complex lipopolysaccharide (LPS) constitutive of the outer membrane of most gram-negative bacteria[50]. LPS is recognized by a receptor complex composed of TLR4, CD14 and MD-2[51,52]. Consequently, using recombinant 14-3-3ε in this study, we wanted to be sure that the effects of 14-3-3ε on joint tissues were due to the protein itself and not to endotoxin contamination. Low levels of endotoxins measured by a LAL kit (14-3-3ε contained less than 0.15 ng/ml of LPS, data not shown) and no significant inhibition by the PMB on 14-3-3ε chondrocyte stimulation (Fig. S2 supplemental data) and 14-3-3ε macrophages stimulation (data not shown) confirmed the proper effect of 14-3-3ε recombinant protein. Moreover, in our previous study[31], we demonstrated that immunodepletion and blocking of 14-3-3ε in conditioned media of compressed osteoblasts inhibited its catabolic effect on chondrocytes confirming that 14-3-3ε itself is involved in the establishment of a procatabolic phenotype in chondrocytes.

Thus, although we cannot rule out that a small component of the effects observed herein were due to endotoxin contamination, we are confident that the cellular responses resulted from the activity of 14-3-3ε protein itself.

Taken together, our results designate 14-3-3ε as a novel alarmin for further exploration in OA for either therapeutic or prognostic purposes.

Contributions

- Conception and design: MM, FB, CJ, FP

- Collection and assembly of data: MM, LS, AR, AP, MN, CR

- Analysis and interpretation of the data: MM, CJ, FB

- Drafting of the article: MM, CJ, FB,

- Critical revision of the article for important intellectual content: MM, CJ, FB, FP, GA-L,

- Final approval of the article: MM, LS, MN, FP, CR, AR, AP, GA-L, FB, CJ

Role of the funding source

Conflict of interest

No potential conflicts of interest relevant to this article were reported. All authors disclose any financial and personal relationships with other people or organizations that could potentially and inappropriately influence their work and conclusions.

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Figure legends

Fig. 1: Stimulation of MCP1 and IL6 protein release by synovium explants from OA patients with 14-3-3ε.

Synovium explants from OA patients were incubated with medium in the presence or absence of 14-3-3ε (1 µg/ml) for 24 hours. A, B: Protein levels of IL6 (A) (n=5) and MCP1 (B) (n=5) released by human synovium explants treated with the control or 14-3-3ε were measured using the enzyme-linked immunosorbent assay (ELISA). Bars show the mean ± s.e.m. (Wilcoxon test) *P<0.05; **P<0.01; ***P<0.001, ns, not significant.

Fig. 2: Stimulation of IL6 and MCP1 expression and protein release in response to 14-3-3ε in primary cultures of human fibroblast-like synoviocytes.

Synovium explants from OA patients were digested and seeded in 6-well culture plates until confluence. The primary cultures were then stimulated with the control medium in the presence or absence of recombinant 14-3-3ε (10 µg/ml) for 24 hours. A,B: Total RNA was extracted, and mRNA levels of IL6 (A) (n=6) and MCP1 (B) (n=6) were determined by qRT-qPCR. Protein levels of IL6 (C) (n=6) and MCP1 (D) (n=6) in cell supernatants were measured by ELISA. Bars show the mean ± s.e.m. (Wilcoxon test) *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

Fig. 3: Stimulation of IL6, MCP1 and CD38 mRNA expression and IL6, MCP1, and TNFα protein release in human macrophages (derived from the THP1 cell line).

Stimulation of macrophages (THP1 cells) was performed using control medium or 14-3-3ε (1 μg/ml) treatment. A,B,C: Total RNA was extracted, and mRNA levels of IL6 (A) (n=5), MCP1 (B) (n=5), and CD38 (C) (n=5) were determined by qRT-PCR. D, E, F: Protein levels of IL6 (D) (n=5), MCP1 (E) (n=5) and TNFα (F) (n=5) in cell supernatants were measured by ELISA. Bars show the mean ± s.e.m. (Mann Whitney test) *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

Fig. 4: Stimulation of IL6 and MCP1 protein release by bone marrow macrophages from WT, TLR2 or TL4 KO mice in response to 14-3-3ε treatment.
Stimulation of bone marrow macrophages (BMM) were performed using control medium or 14-3-3ε (1 μg/ml) treatment. Protein levels of IL6 (A) (n=4) and MCP1 (B) (n=4) in cell supernatants were measured by ELISA. Bars show the mean ± s.e.m. (Two way ANOVA with a Bonferroni post test) *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

Fig. 5a: Involvement of TLR2 and TLR4 in 14-3-3ε-induced MMP-3 (degradative enzyme) release by murine articular chondrocytes.
Murine articular chondrocytes were treated with TLR2, TLR4 blocking antibodies or the pharmacologic inhibitor OxPAPC for 15 minutes and then stimulated with recombinant 14-3-3ε (1 μg/ml) for 24 h. (A, B, C) Total RNA was extracted, and mRNA levels of MMP-3 were determined by qRT-PCR to examine the inhibitory effects of anti-TLR4 (A) (n=5), anti-TLR2 (B) (n=5), and OxPAPC (C) (n=5). (D, E, F) Protein levels of MMP-3 in cell supernatants were measured by ELISA to examine the inhibitor effects of anti-TLR4 (D) (n=5), anti-TLR2 (E) (n=5), and OxPAPC (F)

(n=5). 14-3-3ε-stimulated cells released an average of 350 ng/ml of MMP-3. Bars show the mean values with 95% confidence intervals (One-way ANOVA with a Bonferroni post test) *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

Fig 5b: Involvement of TLR2 and TLR4 in 14-3-3ε-induced IL-6 (pro-inflammatory cytokine) release by murine articular chondrocytes.

Murine articular chondrocytes were treated with TLR2, TLR4 blocking antibodies or the pharmacologic inhibitor OxPAPC for 15 minutes and stimulated with recombinant 14-3-3ε (1 µg/ml) for 24 h. (G, H, I) Total RNA was extracted, and mRNA levels of IL6 were determined by qRT-PCR to examine the inhibitory effects of anti-TLR4 (G) (n=5), anti-TLR2 (H) (n=5), and OxPAPC (I) (n=5). (J, K, L) Protein levels of IL6 in cell supernatants were measured by ELISA to examine the inhibitory effects of anti-TLR4 (J) (n=5), anti-TLR2 (K) (n=5), and OxPAPC (L) (n=5). 14-3-3ε-stimulated cells released an average of 2 ng/ml of MMP-3. Bars show the mean values with 95% confidence intervals (One-way ANOVA with a Bonferroni post test) *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

Fig. 6: Stimulation of IL6 mRNA expression and Il6, MMP3 and MMP13 protein release by murine articular chondrocytes from WT, TLR2 or TL4 KO mice in response to 14-3-3ε treatment.

Stimulation of murine chondrocytes was performed using control medium or 14-3-3ε (1 µg/ml) treatment. Total RNA was extracted, and mRNA levels of IL6 (B) (n=4), MMP3 (C) (n=4) and MMP13 (D) (n=4) were determined by qRT-PCR. Protein levels of IL6 (A) (n=4) in cell supernatants was measured by ELISA. Bars show the mean ± s.e.m. (Two way ANOVA post test)*P<0.05; **P<0.01; ***P<0.001; ns, not significant.

1

2 **Supplemental data:**

3 **Fig. S1:** Stimulation of M1 markers (iNOS, CD38, IL-1β, TNFα) and decrease of M2

4 marker (EGR2) in response to 14-3-3ε treatment murine bone marrow macrophages.

5 Murine BMM were stimulated with 14-3-3ε (1 µg/ml). Total RNA was extracted and

6 mRNA levels of CD38 (A) (n=5), iNOS (B) (n=5), IL-1β (C) (n=5), TNFα (D) (n=5) and

7 EGR2 (E) (n=5) were determined by qRT-PCR. Bars show the mean ± s.e.m. (Mann

8 Whitney test) *P<0.05; **P<0.01; ***P<0.001; ns: not significant. mRNA expression

9 levels of CD38, iNOS, IL-1β and TNFα showed significant fold increase of 28; 216;

10 13 and 7.3 respectively compared with the controls which show the property of 14-3-

11 3ε to induce the expression of BMM type M1 genes markers. Moreover, mRNA

12 expression levels of EGR2, a M2 phenotype marker, was significantly reduced (5.3-

13 fold decrease) after a 14-3-3ε stimulation compared to the BMM controls.

14

15 **Fig. S2:** Assessment of 14-3-3ε recombinant protein proper effect by inhibiting

16 endotoxins with polymyxin B on murine articular chondrocytes.

17 Murine articular chondrocytes cultures were stimulated with 14-3-3ε (1 µg/ml) or LPS

18 (10 ng/ml) with or without PMB (30 µg/ml). Total RNA was extracted and mRNA

19 levels of MMP13 was determined by qRT-PCR (A) (n=3). Protein level of MCP1 in

20 cell supernatants was measured by ELISA (B) (n=3). Bars show the mean ± s.e.m.

21 (One-way ANOVA with a Bonferroni post test) *P<0.05; **P<0.01; ***P<0.001; ns: not

22 significant. Treatment with PMB inhibited MMP13 mRNA expression (15% compared

23 to 14-3-3ε stimulation), MCP1 release (1% compared to 14-3-3ε stimulation) whereas

24 MMP13 mRNA expression and MCP1 protein release in response to LPS stimulation

1   were totally inhibited by PMB. This result show that recombinant 14-3-3ε has its self-
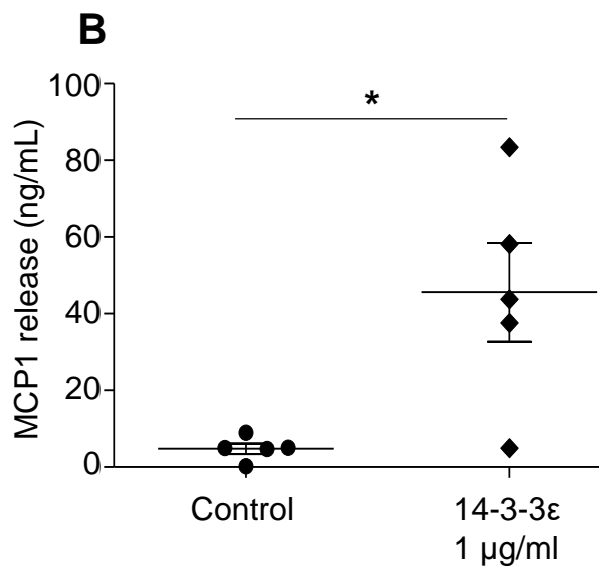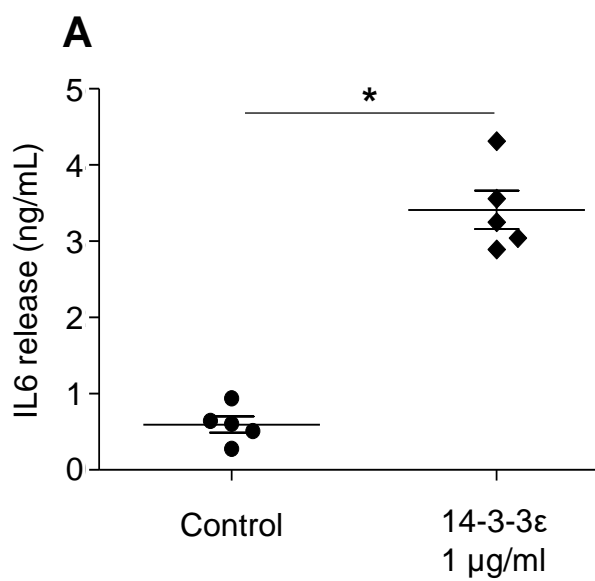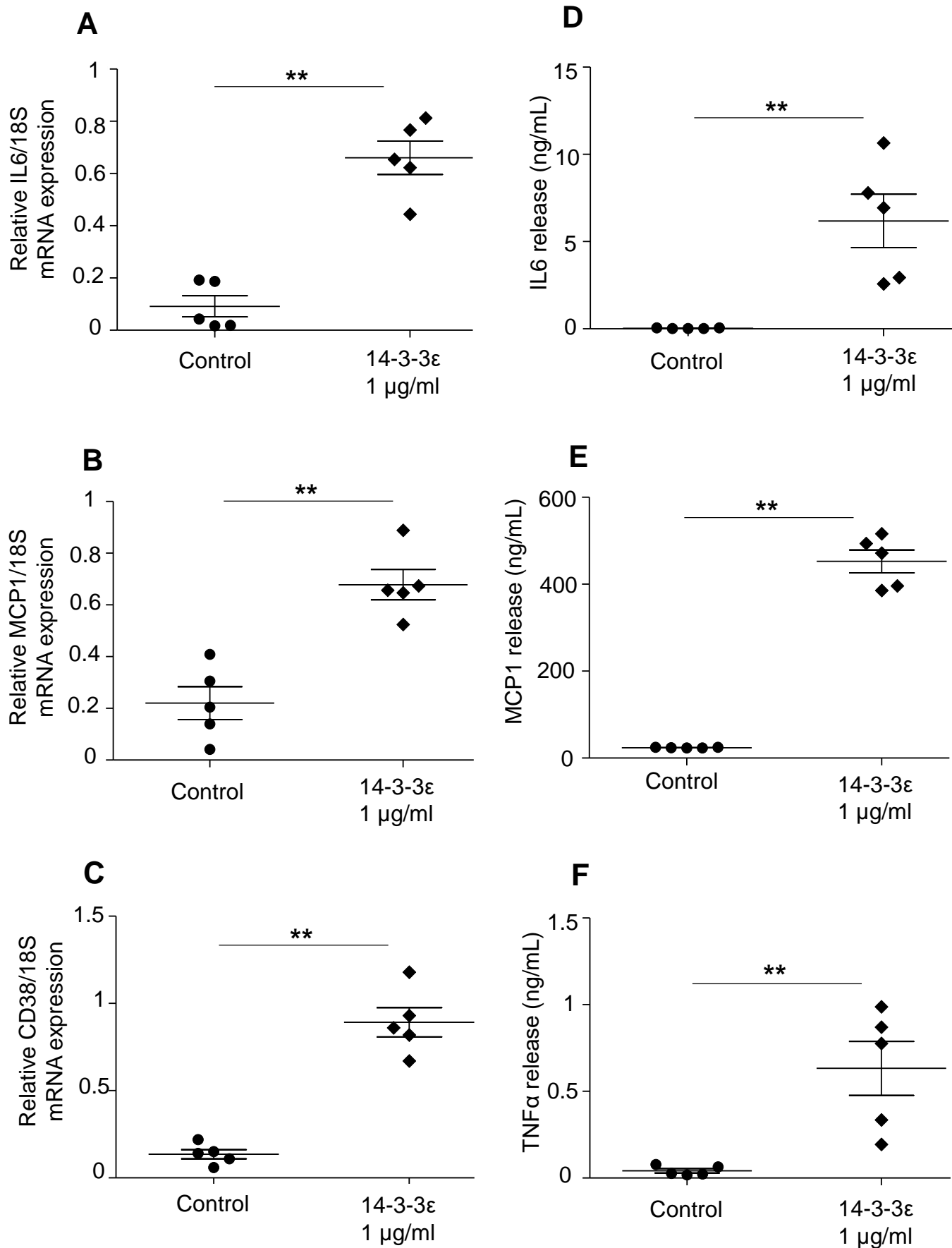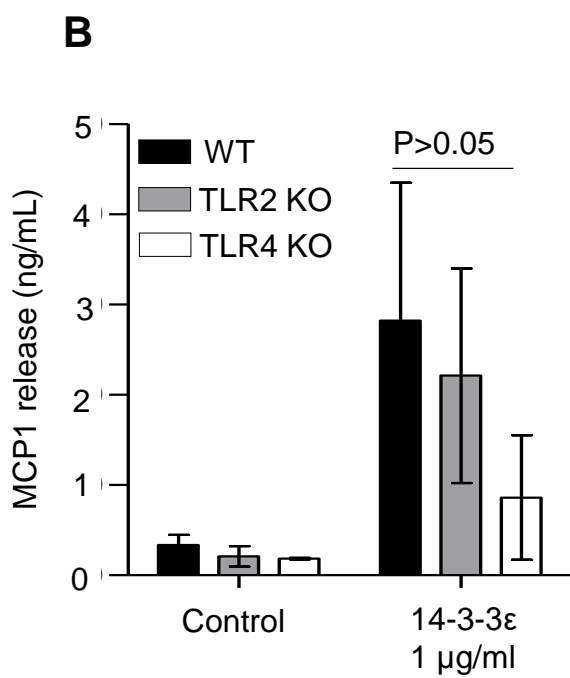
2   effect independent from endotoxins.

Figure 1

Figure 2

A

B

C

D

Figure 3

Figure 4

A

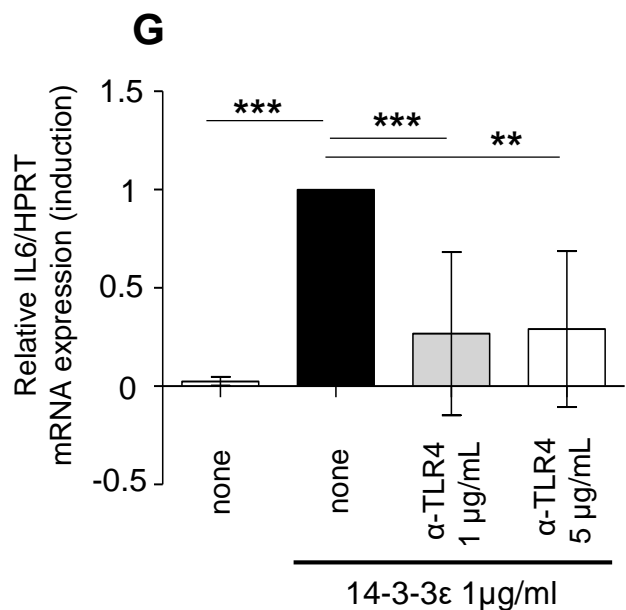
B

Figure 5b

Figure 6

A

B

C

D

Supplemental data
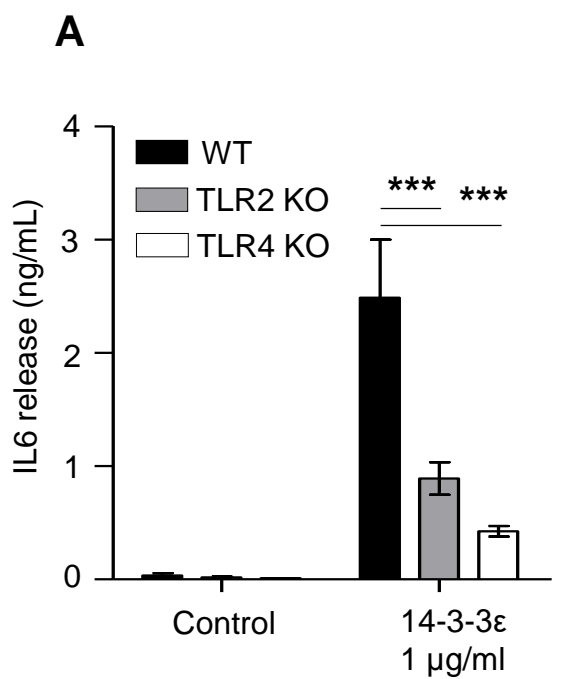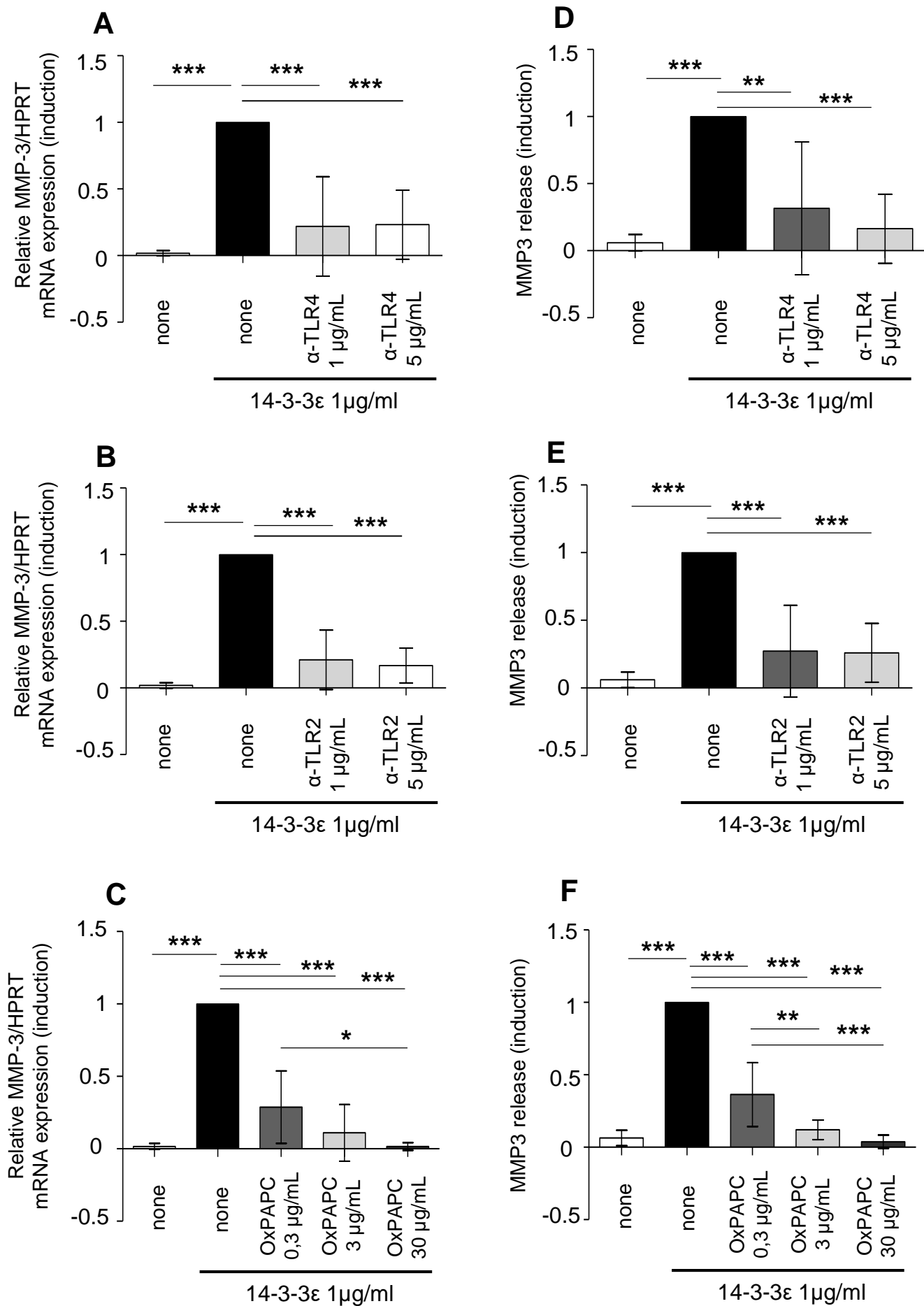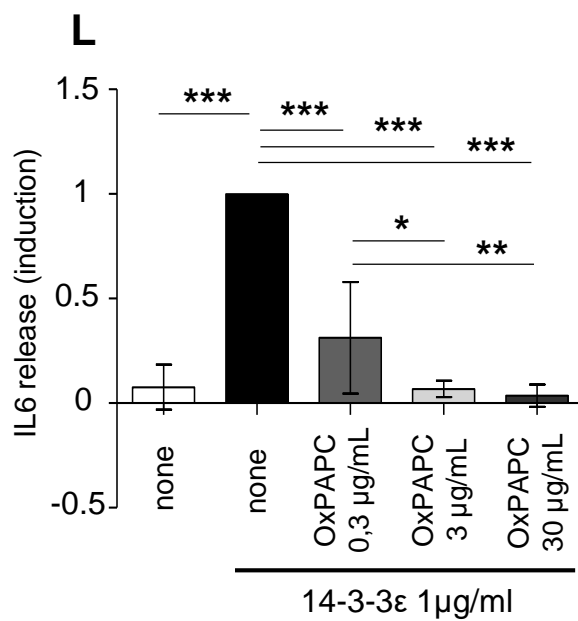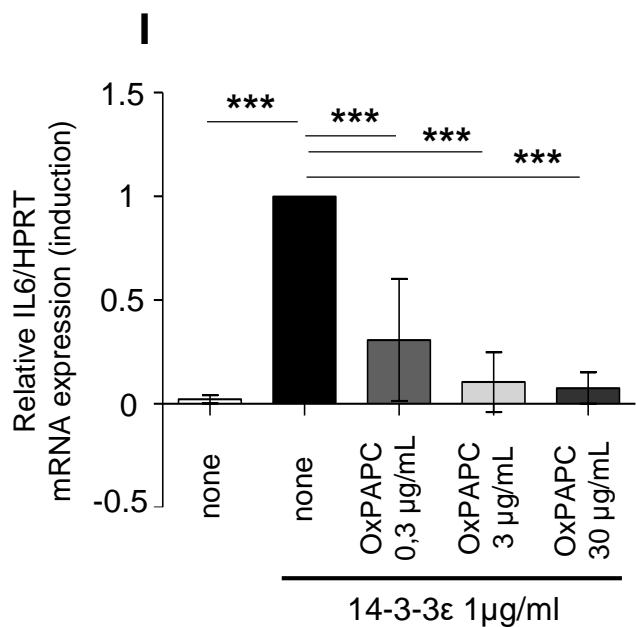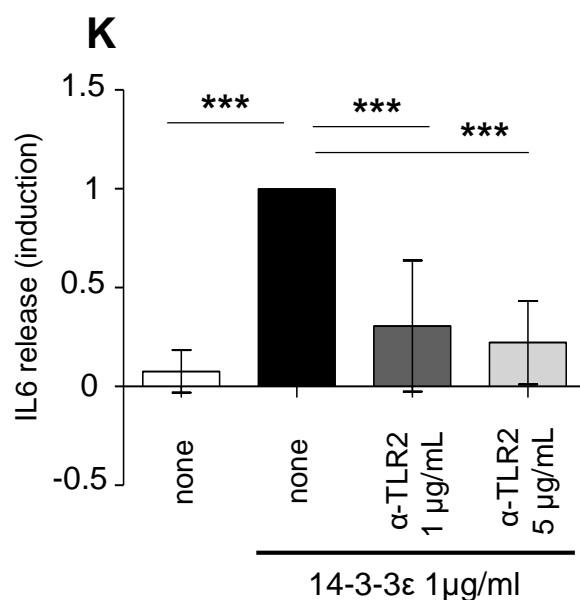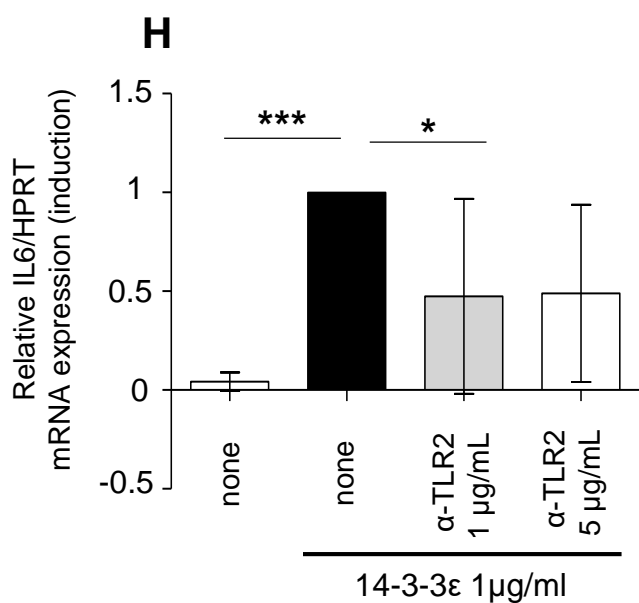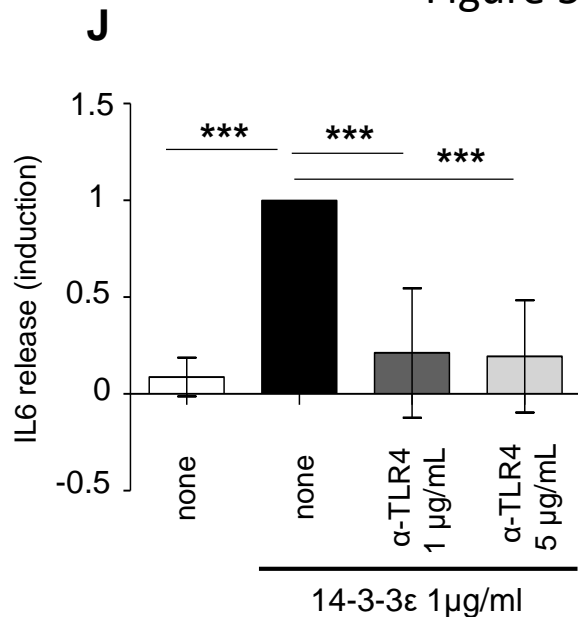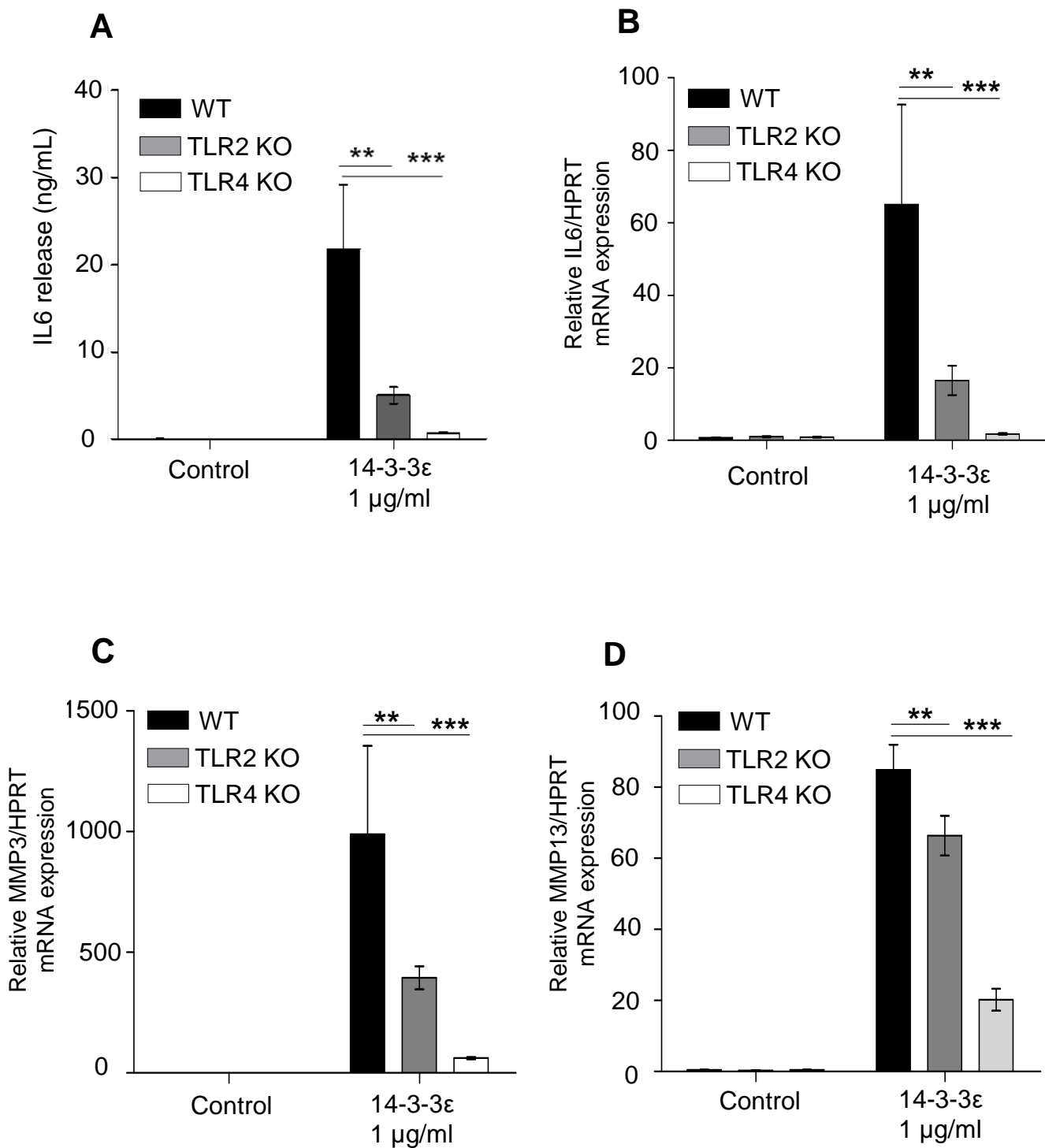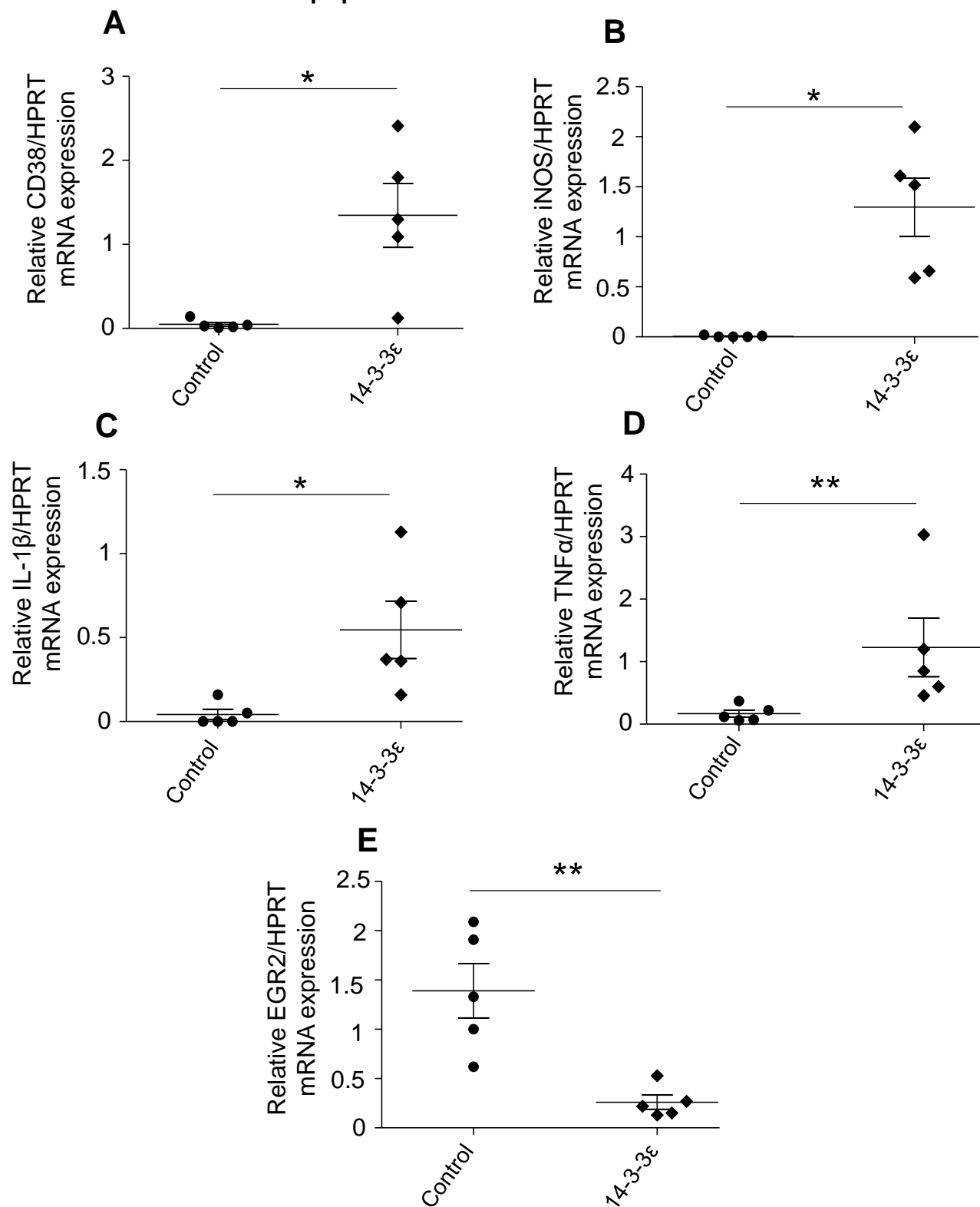

Fig. S1: Stimulation of M1 markers (iNOS, CD38, IL-1β, TNFα) and decrease of M2 marker (EGR2) in response to 14-3-3ε treatment murine bone marrow macrophages. Murine BMM were stimulated with 14-3-3ε (1 μg/ml). Total RNA was extracted and mRNA levels of CD38 (A) n=5, iNOS (B) n=5, IL-1β (C) n=5, TNFα (D) n=5 and EGR2 (E) n=5 were determined by qRT-PCR. Bars show the mean ± s.e.m. (Mann Whitney test) *P<0.05; **P<0.01; ***P<0.001; ns : not significant.
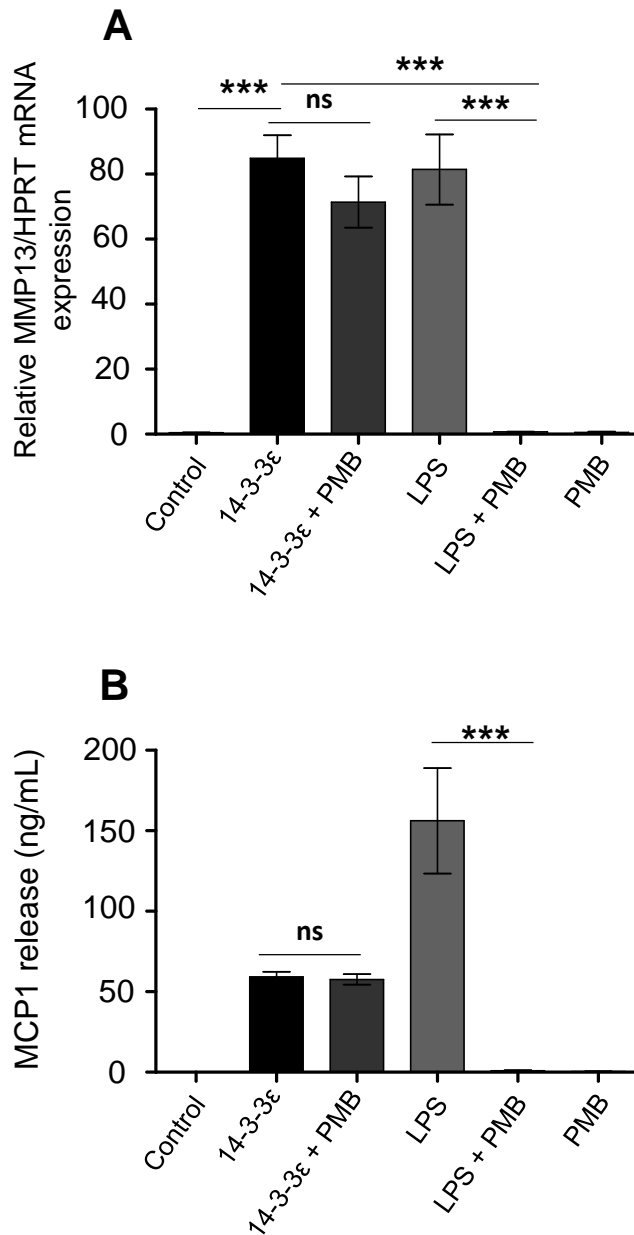
Fig. S2: Assessment of 14-3-3ε recombinant protein proper effect by inhibiting endotoxins with polymyxin B on murine articular chondrocytes.

Murine articular chondrocytes cultures were stimulated with 14-3-3ε (1 μg/ml) or LPS (10 ng/ml) with or without PMB (30 μg/ml). Total RNA was extracted and mRNA levels of MMP13 was determined by qRT-PCR (A) n=3. Protein level of MCP1 in cell supernatants was measured by ELISA (B) n=3. 1% for MCP1 release and 15% for MMP13 mRNA expression, as compared to 100% inhibition between LPS and LPS+PMB in the cellular response, but the major effect of 14-3-3ε was retained even in the presence of polymyxin. (One-way ANOVA with a Bonferroni post test) *P<0.05; **P<0.01; ***P<0.001; ns : not significant.

Species	Name	Forward primer (5'→3')	Reverse primer (5'→3')
Murine	MMP-3	TGAAAATGAAGGGTCTTCCGG	GCAGAAGCTCCATACCAGCA
	MMP-13	GATGGCACTGCTGACATCAT	TGTAGCCTTTGGAACTGCTT
	IL6	GTCACAGAAGGAGTGGCTA	AGAGAACAACATAAGTCAGATACC
	MCP-1	CATCCACGTGTTGGCTCA	GATCATCTTGCTGGTGAATGAGT
	HPRT	AGGACCTCTCGAAGTGT	ATTCAAATCCCTGAAGTACTCAT
	CD68	AAGATGTTCACCCTGGAGGA	ACTCCAATGTGGGCAAGAGA
	iNOS	AAGGTGAGCTGAACGAGGAG	GGAGCCTTTAGACCTCAACAGA
	IL-1β	GGGCCTCAAAGGAAAGAATC	TCCACTTTGCTCTTGACTTCTATC
	TNFα	CAAGGACTCAAATGGGCTTTCCG	TCTGTGAGGAAGGCTGTGCATTG
	EGR2	CTACCCGGTGGAAGACCTC	AATGTTGATCATGCCATCTCC
Human	18S	GCAATTATTCCCCATGAACG	GGGACTTAATCAACGCAAGC
	IL6	CAATGAGGAGACTTGCCTGG	GCACAGCTCTGGCTTGTTCC
	MCP-1	TCAAACTGAAGCTCGCACTCT	GTGACTGGGGCATTGATTG
	CD38	CCTCACATGGTGTGGTGAAT	TTGCTGCAGTCCTTTCTCC

Table S1 : PCR Primers

95°C: 5min	Denaturation (1 cycle)
Denaturation: 10sec at 95°C	Amplification
Hybridation of primers: 15sec at 60°C	
Elongation: 10sec at 72°C	
95°C during 10sec then 60°C during 30sec	Fusion (1 cycle)
40°C: 30sec	Cooling (1 cycle)

Table S2: RT-qPCR amplification conditions